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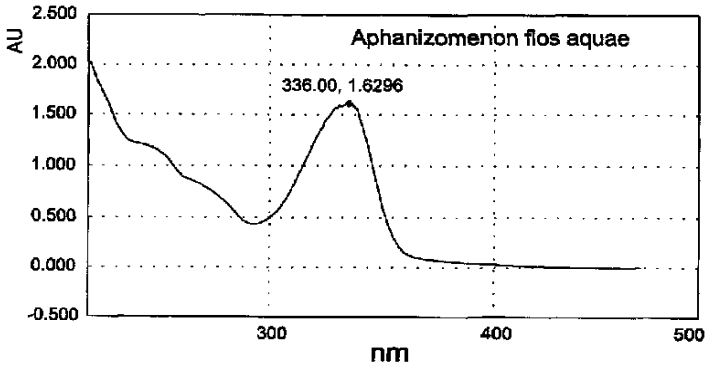
(54) Title: THE UTILIZATION OF NATURAL PIGMENTS FROM LICHENS, CYANOBACTERIA, FUNGI AND PLANTS FOR SUN PROTECTION



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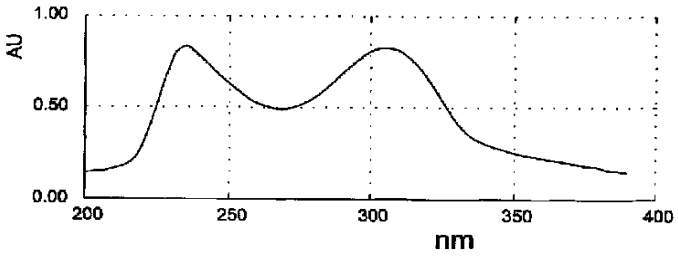

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(57) Abstract: The present invention relates to a natural extract from fungus, cyanobactria, plants, lichens or a mixture thereof having an ultra violet absorbency in the range of 220 nm to 425 nm wherein said extract is obtained by contacting said fungus, cyanobacteria, plant, lichen or a mixture thereof with an C1-7-alcoholic solution. The present invention further relates to a compound of formula I purified from fungus and to cosmetical compositions comprising a natural extract or a compound of formula I.

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THE UTILIZATION OF NATURAL PIGMENTS FROM LICHENS, CYANOBACTERIA, FUNGI, AND PLANTS FOR SUN PROTECTION

FIELD OF THE INVENTION

This invention relates to natural extracts, novel compounds obtained therefrom and their use for medical and cosmetic sun-protection agents.

BACKGROUND OF THE INVENTION

Exposure to ultraviolet radiation (UVR) from the sun plays a causal role in acute and chronic skin damage such as sunburn, skin cancer, immunosuppression, and photoaging of the skin. These consequences of sun exposure are attracting considerable attention due to an alarming increase in the incidence of sun-related skin cancers. Major culprits of increased sun-related morbidity include changes in life style with more time spent in outdoor recreational activities resulting in significant augmentation in the amount of UVR received and depletion of stratospheric ozone, which is the Earth's protection layer against hazardous radiation. To amend for these dangerous developments, a sun avoidance strategy has been advocated in which the topical application of sunscreens constitutes a cornerstone. However, the increased use of sunscreens raises several concerns: Most sunscreens do not effectively filter out all the detrimental wavelengths of sun light. Second, even though sunscreens prevent sunburn, little is known regarding the threshold or dose-response for UVR-induced effects on other endpoints such as immune suppression and DNA damage. Finally, there is increasing body of evidence that presently used topical sunscreens might undergo UV-induced photooxidation and form potentially toxic metabolites.

Commercial sunscreen formulations make use of both organic and inorganic agents as the components of the formulation. Organic sunscreens have been the mainstay of sunscreen formulation for decades. They are classified as derivatives of anthranilates, benzophenones, camphors, cinnamates, dibenzoylmethanes, p–aminobenzoates or salicylates. These aromatic compounds absorb a specific portion of the UVR spectrum that is generally re-emitted at a less energetic, longer wavelength, or used in a photochemical reaction. The organic sunscreens are almost always used in combination in order to provide a high sun protection factor (SPF) and to broaden the absorption spectrum. Inorganic sunscreens, containing either zinc oxide or titanium dioxide, are becoming increasingly popular, mainly due to their ability to filter longer UVA wavelengths. The inorganic particles block radiation by scattering and absorbing incident photons.

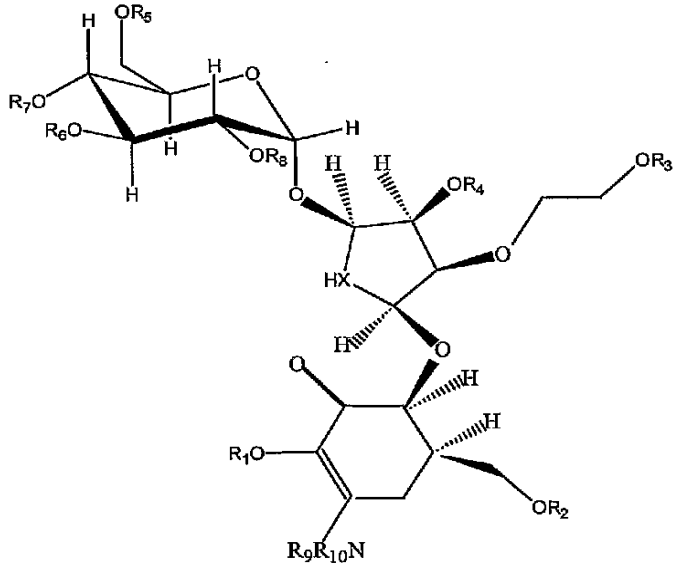
Search for a new generation of sunscreens stems from the various drawbacks the present sunscreen agents possess, including photo- and nonphotoinduced skin sensitivity and photogenotoxicity. Naturally occurring UV filters in the form of pigments are abundant and might constitute attractive candidates for new effective and nontoxic sunscreens. In addition to melanin and flavonoides, they include scytonemins found in cyanobacteria with a recently elucidated structure (Proteau et al (1993) Experimentia 49:825-829). This pigment, the first shown to be an effective photostable UV shield in prokaryotes, is a dimeric molecule of indole and phenol subunits. The scytonemin absorbs strongly and broadly in the spectral region of 325-425 nm (UVA) but also has an absorption in the UVB (280-320 nm) and UVC (<250 nm) regions (US patent No. 5,461,070). Mycosporine is another family of water-soluble, ultra violet-absorbing metabolites found in cyanobacteria with an UV absorption peak in the UVB range. The elucidated structure of mycosporine is cyclohexenone chromophore conjugated with the nitrogen of an amino acid or an amino alcohol. A variety of specific mycosporin amino acids were identified and their distribution in various groups has been described (Karentz et al. (1991) Marine Biology 108, 157-166).

SUMMARY OF THE INVENTION

The present invention is based on the fact that extracts from various natural sources and novel compounds obtained therefrom and their derivatives, absorb efficiently ultra violet radiation and thus may be used as effective sun-protection agents.

Thus the present invention relates to novel natural extracts isolated from fungus, cyanobacteria, plants, lichens or a mixture thereof having ultra violet absorbency in the range between 220 nm and 425 nm. The extraction is done by contacting said fungus, cyanobacteria, plants, lichens or a mixture thereof with a C_{1-7}-alcoholic solution. The alcoholic solution may be an aqueous solution or comprise a hydrophobic organic solvent. The fungus is *Collema* associated fungus, the plant is chosen from the group comprising of *Pecan* nut, *Cichorium endivia*, *Eriobotrya* nut or mixtures thereof, the lichen is *Xanthoria* and the cyanobacteria is chosen from the group consisting of *Spirulina* or *Aphanizomenon* or mixture thereof.

The invention further relates to a compound extracted and isolated from *Collema lichens* and its derivatives of formula (I):



(I)

wherein R^1-R^8 which may be the same or different are selected from the group comprising of hydrogen, a C_1-C_{10}-alkyl or acyl group; R^9 and R^{10} which may be the same or different are selected from the group comprising of C_1-C_{10}-alkyl, aryl, hydrogen or an acyl group; and X is NR, oxygen or sulfur, wherein R is hydrogen, alkyl or aryl.

The invention further relates to cosmetic formulations providing protection for skin from the hazardous effects of ultra violet (UVA and UVB) irradiation, comprising an effective amount of an alcoholic extract from fungus, cyanobacteria, plant, lichen, a mixture thereof or an effective amount of a compound of formula (I). Such cosmetic formulations may further comprise at least one additional sun-protecting agent.

The invention still further relates to the use of an effective amount of the extract of the present invention or of a compound of formula I, optionally together with at least one additional sun-protecting agent for the preparation of a sunscreen formulation providing protection from ultra violet (UVA and UVB) irradiation.

BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting example only, with reference to the accompanying drawings, in which:

Figs. 1A and **1B** show the ultra violet spectrum of extracts from the fungus *Aphanizomenon flos aquae* (1A) and *Collema* associated fungus *(CAF)* (1B).

Figs. 2A and **2B** show the ultra violet spectrum of extracts from *Pecan* nut (2A) and from *Cichorium endivia* subsp. *Divaricatum* (2B).

Figs. 3A, 3B and **3C** show the ultra violet spectrum of extracts from *Eriobotrya* nuts (3A), from *Spirulina* (3B) and from *Xanthoria* (3C).

Fig. 4 shows results from *in vivo* testing of the efficacy of protection of skin of an arm exposed to U.V. irradiation when covered with lotion comprising of (1) *Collema* associated fungus; (2) *Cichorium*; (3) *Eriobotrya*; (4) *Pecan*; and (5) olive oil (serving as control).

Figs 5A and **5B** show the ultra violet spectrum in the range of 290 nm to 400 nm taken in increments of 5 nm of extracts *Spirulina* (5A) and from *Aphanizomenon flos aquae* (5B) showing the UVA/UVB ratio.

Figs 6A and **6B** show the ultra violet spectrum in the range of 290 nm to 400 nm taken in increments of 5 nm of two comparative samples "nivea 3 star" (6A) and "boots soltan 4 star" showing the UVA/UVB ratio.

Fig. 7 shows the ultra violet spectrum of the purified compound (compound I) isolated from the extract of *Collema lichens*.

Fig 8 is a 1-Dimensional NMR spectrum of the purified compound **I** together with its chemical formula.

Figs. 9A and **9B** show the Mass Spectrum of the purified compound **I**. Fig. 9A shows the Time of Flight Electron Spray Ionization (TOF ESI). Fig. 9B is a simulation of the proposed molecular peak at 497.026 corresponding to $C_{19}H_{32}N_2O_{13}$ (MH).

Figs 10A and **10B** show the *in vitro* ultra violet (UVB) protection obtained by the compound of formula I in terms of survival of cultured human keratinocytes (10A) and the protection in terms of pyrimidine dimer formation in cultured human keratinocytes (10B).

Figs. 11A$_{(I,II,III)}$-D$_{(I,II,III)}$ show the *in vitro* ultra violet protection in terms of survival of cultured human keratinocytes (I), the protection in terms of immunosuppression as measured by expression of IL-6 mRNA (II) and the protection in terms of pyrimidine dimer formation in cultured human keratinocytes (III) after ultraviolet (UVB) radiation. Protection was measured with the natural extracts of: CAF (**11A**), *Cichorium* (**11B**), *Pecan* (**11C**) and *Eriobotrya* (**11D**).

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described with reference to some non-limiting specific embodiments. The invention will first be illustrated in reference to the attached drawings to be followed by a more detailed description below.

Figs. **1A** and **1B** show the ultra violet spectrum of the extracts obtained from the cyanobacteria *Aphanizomenon flos aquae* and from the fungal part of the *Collema*, hereinafter termed *Collema* associated fungus (CAF). These two extracts posses a strong absorption in the ultra violet region, the former at 336 nm and the latter at 250nm and 311 nm. Figs **2A** and **2B** show the ultra violet spectrum of the extracts obtained from the plants *pecan* and *Cichorium endivia* showing an ultra violet absorption at 306 nm and 283 nm, respectively. Figs **3A**, **3B** and **3C** show the ultra violet spectrum of the extracts obtained from *Eriobotrya* nuts, from the cyanobacteria *Spirulina* and from lichen *Xanthoria* showing ultra violet absorption at 306 nm, 422 nm and 319 nm, respectively. Fig. **4** shows the *in vivo* efficacy of protection of the skin in the volar forearm exposed to ultra violet irradiation after the arm is covered with a lotion comprising the alcoholic extracts from (1) CAF (2) *Cichorium*, (3) *Eriobotrya* (4) and *Pecan*, all dissolved in olive oil and (5) olive oil taken as a control. It is clearly shown that while the area covered with olive oil turned red upon the ultra violet exposure, the four confined areas where the skin was covered with lotions comprising one of the extracts of the present invention, the skin is unharmed. Such a comparison clearly demonstrates the sun-protecting effect of the extracts.

Figs **5A** and **5B** show the UVA/UVB ratio of the extracts obtained from the two cyanobacteria, *Spirulina* and *Aphanizomenon*. The figures clearly show that the UVA/UVB ratio of the two cyanobacteria extracts is 0.9-0.95. Figs **6A** and **6B** show the UVA/UVB ratio of two commercial sun-protecting lotions (used herein as control) demonstrating a lower UVA/UVB ratio of 0.7 in figure 6A and 0.88 in figure 6B. **Fig. 7** shows the ultra violet spectrum of a purified compound isolated from *Collema lichens*. The spectrum is similar to that of the crude CAF extract (figure 1A), thus demonstrating that this compound is the active material responsible for the ultra violet absorbency of the CAF extract. The chemical formula of the purified compound and its complex 1D-Nuclear Magnetic Resonance spectrum are given in **Fig. 8**. **Fig. 9** further shows the mass spectrum of the purified compound confirming its molecular formula as deduced by NMR. **Fig.**

10 shows the effective *in vitro* activity against ultra violet irradiation (UVB) of the compound purified from *Collema lichens* (compound of formula I). The activity is demonstrated by means of survival of cultured human keratinocytes coated by the purified compound and irradiated by ultraviolet radiation and the protection in terms of pyrimidine dimer formation in cultured human keratinocytes after ultraviolet radiation. **Figs 11A-D** further show the effective *in vitro* activity against ultra violet irradiation (UVB) of the extracts obtained from CAF, *Eriobotrya*, *Pecan* and *Cichorium*. The activity is demonstrated by means of survival of cultured human keratinocytes coated by each of these extracts and irradiated by ultraviolet radiation, the protection in terms of pyrimidine dimer formation in cultured human keratinocytes after ultraviolet radiation and by measuring immunosuppression as indicated by the expression of IL-6 mRNA.

The present invention relates to extracts from natural species wherein the extracts have an ultra violet absorbency both in the UVA and UVB regions and their use in cosmetic preparations providing effective sun protection activity. The extracts of the present invention are from microorganisms such as fungus, cyanobacteria, plants, lichens or mixtures thereof. Such microorganisms, plants and lichens live in an environment exposed to strong solar radiation and have developed an effective ultra violet protection system enabling them to be exposed for long periods to sunlight that comprises ultra violet irradiation, with no apparent damage in their function. In particular the present invention concerns C_{1-7}-alcoholic extracts from the fungal part of the *Collema* which is termed *collema* associated fungus (CAF), from the cyanobacteria *Aphanizomenon* and *Spirulina,* from the plants *Pecan* nuts, *Cichorium endivia* subsp. *Divaricatum* - a wild plant, *Eriobotrya* nuts and from the lichen *Xanthoria*. The CAF after isolation from the *Collema* may easily be grown on PDA medium.

The naturally occurring material used as source for the isolation of the extracts of the present invention were grounded and the active material extracted with a C_{1-7}-alcoholic solution. The alcoholic solution may be an aqueous solution or a solution comprising a mixture of alcohol and a hydrophobic organic solvent.

The C_{1-7}-alcohol used in the present invention are straight or branched C_{1-7}-alcohols. The aqueous alcoholic solution comprises H_2O:ROH in a ratio from 50:50 (%) to 5:95 (%). Particular non-limiting examples of the organic solvent used in the present invention are selected from the group consisting of alkyls, chlorinated alkyls, esters, ketones, aldehydes and aromatics. The UV absorbency of each extract is determined. The extracted compounds are very strong UV absorbers that absorb strongly in the spectral region from 220 to 425 nm. Of particular interest is the fact that the two extracts from cyanobacteria, *Spirulina* and *Aphanizomenon* display UVA/UVB ratios of 0.9 to 0.95. Such a UVA/UVB ratio, which is close to unity, reveals that these extracts are materials having effective absorbance activity in the UVA region.

In addition to the alcoholic extracts, an extract from *Collema lichens* was further purified to yield a compound of formula I (R and R_1-R_{10} are hydrogen and X is NH) whose chemical structure was elucidated (see figure 8). The determination of the chemical structure was done using IR, UV and NMR. The IR spectrum revealed the presence of a carbonyl, primary amine and a double bond. These three functional groups are part of the mycosporine skeleton, the aminocyclohexanone ring. The existence of the primary amine group was also confirmed by ninhydrin. Various 1-Dimensional and 2-Dimensional NMR pulse sequences (DEPT, COSY, NOESY, HMBC, HSQC – data not shown) were further used to elucidate the entire structure of the pure isolated extract. From these various NMR experiments it may be deduced that the isolated compound consists of a glucoside residue bonded via pyrrolidine ring to an aminocyclohexanone ring. The pyrrolidine ring, in its meta position, has a further C_2H_4OH chain bonded to the ring via an oxygen. It should be mentioned that aminocyclohexanone moiety is part of the known elucidated structure of mycosporines, one of the naturally occurring pigments found in lichens. However, the presence of the pyrrolidine ring renders the compound a new member of the mycoscopine family.

The naturally occurring compound of formula (I) extracted form *Collema lichens* may easily be derivatized to yield compounds of formula (I) where R^1-R^8

which may be the same or different selected from the group consisting of hydrogen, a C_1-C_{10}-alkyl or acyl group; R^9 and R^{10} which may be the same or different are selected from the group consisting of C_1-C_{10}-alkyl, aryl, hydrogen or an acyl group; and X is NR, oxygen or sulfur, wherein R is hydrogen, alkyl or aryl. The rational of derivatization of the isolated natural compound is that the naturally occurring compound of formula I is hydrophilic, while an effective sun screen compound should be hydrophobic in order for the compound to remain on the skin despite external humidity.

It should however be understood that the use of the extracts or the purified compound or its derivatives of formula I as effective sun protection lotions, may also be by encapsulating them in appropriate encapsulating agent thus rendering their environment hydrophobic and aiding in dispersion. Alternatively they may be used together with at least one additional organic or inorganic sun protecting agent. Non limiting examples of the at least one additional sun protecting agent are derivatives of anthranilates, benzophenones, camphors, cinnamates, dibenzoylmethanes, p–aminobenzoates, salicylates, zinc oxide, titanium dioxide and mixtures thereof.

Evaluation of the sun-protecting activity of the various extracts of the present invention and of the compound of formula I in terms of preventing UVB and/or UVA induced damage as a result of irradiation was assessed both *in vitro* and *in vivo*. Such assessment was done using cell death, immunosuppression, and DNA damage as biological endpoints. *In vivo* determination was done by applying extracts obtained from CAF, *Cichorium*, *Eriobotrya* or pecan dissolved in olive oil on the volar forearm. Olive oil served as the control. Treated skin was totally protected from UVB induced erythema, while olive oil did not show such protection (Fig 4).

In vitro evaluations were done using cultures of the human keratinocyte cell line, HaCaT which were irradiated with 200 mJ/cm^2 (cell death and immunosuppression) or 60 mJ/cm^2 (DNA damage) UVB delivered from a bank of four FS40 fluorescent lamps that emit wavelengths between 280 and 320 nm, with

a peak at 313 nm. The cells were irradiated through a quartz plate on which solutions of the compounds were spread, and harvested immediately (DNA damage) or after 24 hours (cell death). Cell death was evaluated by vital staining, immunosuppression by IL-6 mRNA expression, and DNA damage was assayed with a polymerase chain reaction using primers for pyrimidine dimers and ELISA for staining of the cDNA product. Irradiation through naked quartz plated served as control. These tests confirm in terms of photoprotection the biological activities that were postulated based on the UV absorbing property of the extracts (Figs 11A-D).

Examples

Chemical

Extraction of sun-protecting agents from various natural sources.

Example 1: ***Aphanizomenon flos aquae*** (Upper Klamath Lake,USA).

10gr. of green powder (capsules comprising of *Aphanizomenon Flos Aquae* made by SOLGAR, IL) was transferred into a 500 ml flask connected to a condenser. 250 ml of 75% aqueous Methanol were added, and the solution was boiled for one hour. After reaching room temperature the extract was filtered through a Bichner funnel (Schleicher & Schuell 595 filter papers). The extract (intense green color) was transferred into a 500ml separatory funnel. A mixture of 100 ml CH_2Cl_2 and H_2O was added. The layers separated, where the organic phase remains as an emulsion (green) and transferred into flask I.

The upper aqueous layer (clear and yellow) was transferred to a second separatory funnel and the extraction procedure was repeated again with the same solvent mixture in the presence of NaCl. After separation, the organic layer was transferred into flask II. The aqueous layer appeared as clear and yellow, and transferred into flask III. The organic solvent's residues from flask III were evaporated, and the water was lyophilized till dryness. 4.326gr. of a pale yellow powder was obtained.

A sample of the powder was redissolved in methanol and checked for its UV absorbance. A UVA peak is observed at 336 nm (**Fig. 1A**).

Example 2: ***Collema* associated fungus *(CAF)*** (one out of four fungus which exist in a Lichen known as *Collema sp.*) The CAF was isolated and easily grown on PDA medium.

138gr. of fresh fungus (black) were transferred into a mortar. 100 ml of 90% aqueous methanol was added and the fungus was crushed by a pestle. The crushed material was transferred into a beaker and heated at 60°C for 3 min. After reaching room temperature the material was filtered through a funnel (Schliecher & Schuell 593 ½ filter papers). The extract was transferred into a 250 ml flask and the solvents (organic and water) were moved till dryness yielding a pale yellow powder.

The powder was dissolved in 100ml H_2O, filtered through cotton and transferred into a separatory funnel. The upper organic phase is colorless, while the lower aqueous phase appears as clear yellow. The aqueous layer was lyophilized to dryness, and 28mg of pale yellow powder were obtained.

A sample of the powder was diluted in 70% aq. methanol and UV absorbance was measured. As a blank we used PDA medium that was dissolved in 70% aq. methanol. A UVB peak at 311 nm (**Fig. 1B**) was found.

Example 3: *Pecan.*

100gr of *Pecan* nuts (purchased on local market in Jerusalem, IL) were homogenized in a high-speed unit and transferred into a 500ml flask connected to a condenser. A mixture of 100 ml of methanol and 100 ml CH_2Cl_2 were added, and solution was boiled for 1 h. After reaching to room temperature the extract was filtered through a Bichner filter. The extract (green-brown) was transferred into a 500 ml separatory funnel. A mixture of 100 ml C_2H_2 and 200ml cold H_2O (with ice) was added. The layers were separated. The organic phase (yellow-bright) was

transferred into flask. The organic layer was dried over $MgSO_4$. The lipid-soluble extract has UV absorbance 294 and 302 nm (**Fig. 2A**).

Example 4: *Cichorium endivia* subsp. *Divaricatum*

500gr dried parts of *Cichorium endivia* subsp. *Divaricatum* (The wild plant *Cichorium endivia* L. subsp. *divaricatum* (Schousb.) P.D. Sell was collected at Sedot Micha, IL) stalks, inflorescences, and roots were separately homogenized in a high-speed unit. Homogenized roots were transferred into a 2000ml flask connected to a condenser. A mixture of 500ml of ethanol and 300ml water were added, the solution was boiled for 6 hrs. After reaching to room temperature the extract was filtered through a Bichner filter. The ethanol-water extract (yellow-brown) was transferred into a 1500ml separatory funnel. A mixture of 300 ml C_2H_2 and 300ml cold H_2O (with ice) was added. The layers were separated. The organic phase remains as an emulsion (brown) and transferred into flask 1 (Fraction No. 1). The methanol-water layer (upper layer) was evaporated *in-vacuo* and the resulting oil dissolved in 50ml dry ethanol (Fraction No 2). To Fraction No 2 was added 50ml (x 3) hexane (Fraction No 3). The layers were separated, and hexane layer was dried over $MgSO_4$. The three fractions gave three different absorption patterns (**Fig. 2B**): Fraction No. 1 has UV absorbance 294 nm. Fraction No. 2 has UV absorbance 280 nm; and Fraction No. 3 has UV absorbance 283 and 312 nm

Example 5: *Eriobotrya* nuts.

100gr of fresh *Eriobotrya* nuts (purchased on local market in Jerusalem) were homogenized in a high-speed unit and transferred into a 500ml flask connected to a condenser. A mixture of 100 ml of methanol and 100 ml CH_2Cl_2 were added, and the solution was boiled for 1hr. After reaching room temperature the extract was filtered through a Bichner filter. The green-brown extract was transferred into a 500ml separatory funnel. A mixture of 100ml C_2H_2 and 200ml cold H_2O (with ice) was added. The layers were separated. The organic phase

(green-brown) was transferred into flask. The organic layer was dried over $MgSO_4$. The lipid-soluble extract has a UV absorbency at 298 nm and 306 nm (**Fig. 3A**).

Examples 6 & 7:

Spirulina and *Xantoria* were extracted by similar methods and their ultra violet spectra are given in Figs. **3B** and **3C**.

Example 8: Extraction of a compound of formula I (R^1-R^{10}= H; and X=NH).

Collema (taken from sun exposed rocks near Jerusalem) were washed from sediments grounded, immersed in an H_2O/C_3OH solution (1:1). The mixture was heated to a temperature of 60°C for 30 minutes and filtered. The biological material was subjected to another two consecutive extraction cycles with the same solvent system. The combined extracts were evaporated, immersed in dichloromethane to separate soluble lipid compounds, water added and the aqueous fraction separated. The water were lyophilized and the residue was redissolved in 90 % CH_3OH/H_2O and the U.V. spectrum of the material was measured. Dry material extracted from *Collema lichens* showed significant U.V. absorption (**Fig. 7**) and was further analyzed for its structure and activity. Thin layered chromatography revealed the presence of several components. Separation of the various components and isolation of the active component having an intense U.V. absorption was done by HPLC (RP_{18}; Gradient elution of from 90 % 0.05 % acetic acid in water: 10 % acetonitrile to 10 % of 0.05% acetic acid in water:90 % acetonitrile; flow rate of 1ml/min). The fraction eluted with RT of 14.1 min. gave the intense U.V absorbance (data not shown).

Example 9: Determining the chemical structure of the extracted compound of formula I.

An infra red spectrum (FTIR) of the extracted material (data not shown) revealed the presence of –OH, -NH and C=O groups (3382, 2936 and 1653 cm^{-1},

respectively). Various 1-dimensional and 2-dimensional Nuclear Magnetic Resonance experiments (DEPT, COSY, HSQC, HMBC, NOESY and TOCSY- not shown) were carried on 400 MHz and 600 MHz instruments in order to elucidate the chemical structure of the extracted compound.

The ^{1}H **(Fig. 8)** and ^{13}C spectrum (not shown) were too complex for an interpretation, but they clearly demonstrated the presence of an anomeric proton and 16 carbons. Partial structure was confirmed by COSY, DEPT, HSQC, HMBC and NOESY experiments. The COSY revealed the existence of a glucoside and three other systems:

The first system consists of four protons assigned as 1'-4', which create a pyrrolidine ring through a withdrawing group (Nitrogen).

The second system is a four protons, named as Xa, Xb, Ya, Yb having the following interactions:

Xa ➔ Xb, Yb

Xb ➔Xa, Ya

Ya ➔Xb, Yb

Yb ➔Xa, Ya

this short chain is connected to a meta position of the pyrrolidine through an oxygen atom.

The third is a five protons system assigned as C-F, which then by all other experiments was found to be a part of an aminocyclohexenone ring.

Carbon number	$\delta^{13}C$ (ppm)	HSQC	$\delta^{1}H$ (ppm)	DEPT	HMBC
1	104.9	1g (d)	4.45	CH	1'
2	79.1	1' (d)	4.02	CH	1g, 3'
3	78.3	3g	3.37	CH	2g, 4g
4	78.1	5g	3.35	CH	
5	75.5	2g (t)	3.24	CH	3g, 5g
6	73.3	C	3.7	CH	

7	72.8	2'	3.84	CH	
8	72.7	4g (t)	3.19	CH	
9	72.2	3'	3.86	CH	X, Y
10	71.9	4' (d)	3.71	CH	D, C
11	71.7	D	3.72	CH	F_b
12	65.4	Y_a, Y_b	3.76,3.6	CH_2	
13	65.4	X_a, X_b	3.82,3.63	CH_2	
14	65.4	F_a, F_b	3.81,3.62	CH_2	
15	64.1	E	3.74	CH_2	
16	63.6	$6g_a$, $6g_b$	3.92,3.57	CH_2	4g

Analysis of the various 1D and 2D NMR experiments give rise to a chemical structure as shown in **Fig. 8**. Such a compound has a chemical formula of $C_{19}H_{32}N_2O_{13}$ and its molecular weight is 496. Confirmation for such a chemical structure and formula was obtained from the Mass Spectrum of purified material. The mass spectrum is shown in **Fig. 9A** as the Time of Flight Electron Spray Ionization (TOF ESI) reveals a molecular peak of 497.026 corresponding to the MH+. **Fig. 9B** further shows a simulation of the proposed molecular peak at 497.198 corresponding to $C_{19}H_{32}N_2O_{13}$.

Biological activity

Example 10: ***In vivo* activity of extracts**

The *in vivo* biological activity was assayed by the ability to prevent UV induced erythema of human skin. Extracts were diluted in olive oil and applied to the volar forearm of a volunteer. Olive oil without extract served as control. Fifteen minutes after application of the extracts dissolved in olive oil and the olive oil to the volar forearm, 2 MED of UVB irradiation was delivered to the treated areas, and the resulting erythema was evaluated after 24 hours. Treated skin was totally protected from UV induced erythema (**Fig. 4**).

Example11: *In vitro* activity of extracts

Evaluation of the biological activity of the various extracts in terms of preventing UVB induced damage was assessed *in vitro* using cell death, immunosuppression, and DNA damage as biological endpoints: Cultures of the human keratinocyte cell line, HaCaT were irradiated with 200 mJ/cm^2 (cell death and immunosuppression) or 60 mJ/cm^2 (DNA damage) UVB delivered from a bank of four FS40 fluorescent lamps that emit wavelengths between 280 and 320 nm, with a peak at 313 nm. The cells were irradiated through a quartz plate on which solutions of the extracts were spread, and harvested immediately (DNA damage) or after 24 hours (cell death). Cell death was evaluated by vital staining, immunosuppression by IL-6 mRNA expression, and DNA damage was assayed with a polymerase chain reaction using primers for pyrimidine dimers and ELISA for staining of the cDNA product. Irradiation through naked quartz plated served as control. The results of the biological activity are summarized in **Table I** and shown in **Figs. 11A$_{(I,II,III)}$-D$_{(I,II,III)}$** where the following may be noted.

Collema associated fungus *(CAF)* at a concentration of 157 $\mu g/cm^2$ totally prevented UVB induced erythema 11A$_{(I)}$ and partially prevented UVB induced cell death and cyclobutane pyrimidine dimer formation 11A$_{(III)}$, but did not prevent IL-6 expression 11A(II).

Cichorium extracts (6 $\mu g/cm^2$) totally prevented UVB induced erythema 11B$_{(I)}$ and partially prevented UVB induced cell death IL-6 expression 11B$_{(II)}$ and cyclobutane pyrimidine dimer formation 11B$_{(III)}$.

Pecan extracts totally prevented UVB induced erythema 11C(I) and partially prevented UVB induced cell death, IL-6 expression 11C$_{(II)}$ and cyclobutane pyrimidine dimer formation 11C$_{(III)}$.

Eriobotrya extracts totally prevented UVB induced erythema 11D$_{(I)}$ partially prevented UVB induced cell death and IL-6 expression 11D$_{(II)}$ but was not effective in preventing cyclobutane pyrimidine dimer formation 11D$_{(III)}$.

Table I:

Extract	Absorbance	UV Protection				
		In vivo	In vitro			
			Concentration	Survival	IL-6	CPD[3]
CAF[1]	311 nm	Yes	157 μg/cm^2	31 %	5%	75 %
Cichorium	283 nm	Yes	6 μg/cm^2	39 %	49 %	69 %
Eriobotrya	305 nm	Yes	Unknown[3]	41 %	65 %	91 %
Pecan	305 nm	Yes	Unknown	89 %	23 %	3%
Aphanizomenon	336 nm	ND[2]	ND	ND	ND	ND
Xantoria	333 nm	ND	ND	ND	ND	ND

[1] Collema-associated fungus

[2] Not determined

[3] Dissoved in oil in undetermined concentration

[4] Cyclobutane pyrimidine dimers

Example 12: Determining UVA/UVB ratio of extracts.

Determining the UVA/UVB ratio is intended to give an indication of the scope of the UV absorbence properties of a test product as part of a "broad spectrum" claim (HW Lim et al. (2001) JAAD 44:505-508).

Using UV transmittance spectrophotometry, UV radiation was directed onto the surface of a smooth quartz glass plate and the quantity of radiation transmitted through the plate was measured. UV transmission through the plate was measured at 5nm increments throughout the UVB and UVA regions (290nm to 400nm) at single discrete regions of the plate to determine 100% transmission.

A roughened quartz glass plate was coated with the appropriate natural extract whose UV absorbency was measured. Coating was done in the following manner. The natural extract was dissolved in water at a concentration of 10mg/mL and the solution was applied in a series of small dots to the roughened quartz glass plate using a micro-pipette and then spread evenly using a gloved finger to achieve a typical uniform application rate of 0.75mg/cm^2 of product. Care was taken to

ensure the formation of a uniform film. The coating was allowed to dry for ca. 10 minutes. Radiation from a U.V. source was directed onto the surface of the coated plate and the quantity of radiation transmitted through the coated plate was measured. UV transmission was measured at 5nm increments from 290nm to 400nm at 6 discrete regions of the plate. This procedure was carried out 3 times for each of the extracts whose ultra violet absorbency was measured. Two internal control containing commercial sun-protecting products agents were tested prior, and after the irradiation extract, for validation purposes. The two internal control products are hereinafter termed "nivea 3 star" (SPF 12) and "boots soltan 4 star" (SPF 4). The transmission measurements obtained before and after the substrate coating at discrete wavelength intervals from 290nm to 400nm was used to give an indication of the protection potential of the product throughout the UVA and UVB regions.

The "critical wavelength" and the UVA protection categories were then determined as shown in the Table below (according to the Boots the Chemists Ltd UVA symbol system based upon an *in vitro* technique previously described by B. L. Diffey and J. Robson (J. Soc. Cosmet. Chem, 40, 127-133, May/June 1989)). A "Critical wavelength" value equal or greater than 370 nm is required for broad-spectrum protection claim. The "Boots" Star method protection categories are summarised in Table II.

Table II

Mean UVA/UVB Protection Ratio	UVA Protection Category
0 - <0.2	Too low for UVA claim
0.2 - < 0.4	Moderate (*)
0.4 - < 0.6	Good (**)
0.6 - < 0.8	Superior (***)
0.8 +	Maximum (****)

The mean and standard deviation UVA/UVB protection ratios based on three measurements done for each substrate, critical wavelength and percentage UVA block calculated for the two extracts and the control compounds are given in Table III.

Table III

Substrate	Mean critical wavelength	Mean UVA/UVB ratio	UVA protection category (Table II)
Nivea 3 star	377	0.64 ± 0.01	Superior (***)
Boots soltan 4 star	380	0.88 ± 0.01	Maximum (****)
Spirulina	388	0.92 ± 0.01	Maximum (****)
Aphanizomenon flos aqua	388	0.95 ± 0.01	Maximum (****)
Nivea 3 star	375	0.68 ± 0.01	Superior (***)
Boots soltan 4 star	380	0.89 ± 0.01	Maximum (****)

Example 13: *In vitro* activity of the compound of formula I (purified from *Collema lichens*).

A compound of formula I at a concentration between 0.5 to 3 mg/cm^2 was spread on a quartz plate, which covered the dish, containing the cultured cells. Four FS-40 fluorescent lights radiating at wavelengths between 280 and 320 nanometers served as the source of UVB. These lamps were placed at a distance of 20 cm from the quartz plate. Under the same conditions of irradiation a cultured cell preparation was irradiated with no protection, i.e. naked quartz plate.

A: Cells survival.

Fig. 10A shows the cell survival after 200mJ/cm^2 UVB irradiation. UVB protection was offered in a dose-dependent manner, since 74 % of the cells survived when cells were protected with 3 mg/cm^2 of the compound whereas only 43 % survived with 0.16 mg/cm^2 of the compound. Only 20 % of the cells survived when irradiated through the naked quartz plate.

B: DNA damage

Fig. 10B shows generation of pyrimidine dimers after 60 mJ/cm^2 UVB irradiation through quartz plates covered with 6 mg/cm^2 of the formula (I) compound, commercial sunscreen or naked quartz plate. Non-irradiated cells showed a low background of pyrimidine dimers. Plates covered with the formula (I) compound offered around 80 % protection compared to cells irradiated through the naked quartz plate.

Although the invention has been described in conjunction with specific embodiments, it is evident that many alternatives and variations will be apparent to those skilled in the art in light of the foregoing description. Accordingly, the invention is intended to embrace all of the alternatives and variations that fall within the spirit and scope of the appended claims.

CLAIMS:

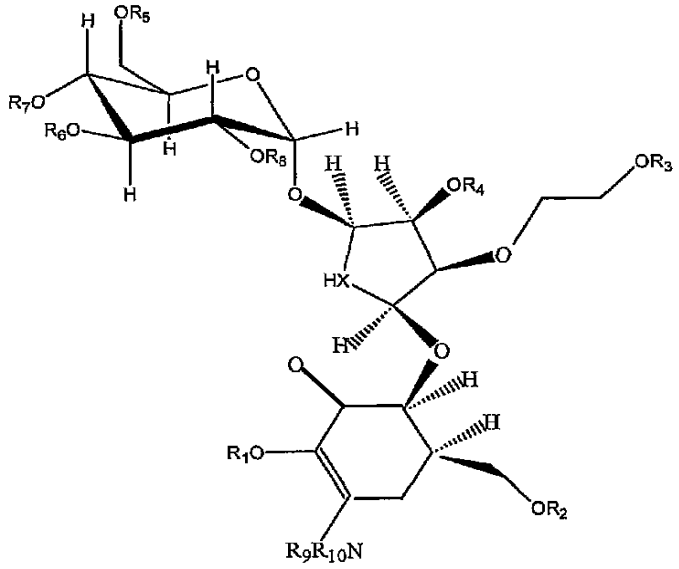
1. A natural extract from fungus, cyanobactria, plants, lichens or a mixture thereof having an ultra violet absorbency in the range of 220 nm to 425 nm wherein said extract is obtained by contacting said fungus, cyanobacteria, plant, lichen or a mixture thereof with an C_{1-7}-alcoholic solution.

2. A natural extract according to claim 1, wherein the alcoholic solution is an aqueous alcoholic solution comprising H_2O:ROH in a ratio of from 50:50(%) to 5:95(%).

3. A natural extract according to claim 1, wherein the alcoholic solution comprises a hydrophobic organic solvent selected from the group consisting of alkyls, chlorinated alkyls, esters, ketones, aldehydes or aromatics.

4. A natural extract according to claim 1, wherein the fungus is *Collema* associated fungus, the plant is chosen from the group comprising of *Pecan* nut, *Cichorium endivia*, E*eriobotrya* nut or mixtures thereof, the lichen is *Xanthoria* and the cyanobacteria is chosen from the group consisting of *Spirulina* or *Aphanizomenon* or mixture thereof.

5. A compound of formula (I)



(I)

wherein R^1-R^8 which may be the same or different are selected from the group comprising hydrogen, a C_1-C_{10}-alkyl or acyl group; R^9 and R^{10} which may be the same or different are selected from the group comprising of C_1-C_{10}-alkyl, aryl, hydrogen or an acyl group; and X is NR, oxygen or sulfur, wherein R is hydrogen, alkyl or aryl.

6. A compound according to claim 1 wherein R^1-R^8 are hydrogen, R^9 and R^{10}, which may be the same or different are selected from the group comprising of C_1-C_5-alkyl, aryl, hydrogen or an acyl group and X is NH.

7. A compound according to claim 1 wherein R, R_1 – R_{10} are hydrogen and X is NH.

8. A cosmetic formulation for providing protection from ultra violet irradiation comprising an effective amount of an extract of claims 1 to 4 or of an effective amount of a compound of formula (I) of claim 5 together with suitable excipients.

9. A cosmetic formulation according to claims 8 further comprising an additional sun-protecting agent.

10. A cosmetic formulation according to claim 9, wherein said additional sun-protecting agent is chosen from the group comprising of derivatives of anthranilates, benzophenones, camphors, cinnamates, dibenzoylmethanes, p–aminobenzoates, salicylates, zinc oxide, titanium dioxide and mixtures thereof.

11. A cosmetic formulation according to claim 8, wherein said ultra violet irradiation is UVA or UVB.

12. Use of an effective amount of an extract of claims 1 to 4 or of an effective amount of a compound of formula (I) of claim 5 for the preparation of a cosmetic formulation for providing protection for ultra violet irradiation.

13. Use according to claim 12, further comprising an additional sun-protecting agent.

14. Use according to claim 13, wherein said additional sun-protecting agent is chosen from the group comprising of derivatives of anthranilates, benzophenones,

camphors, cinnamates, debanzoylmethanes, p–aminobenzoates, salicylates, zinc oxide, titanium dioxide and mixtures thereof.

15. Use according to claim 12 wherein said ultra violet irradiation is UVA or UVB.

5

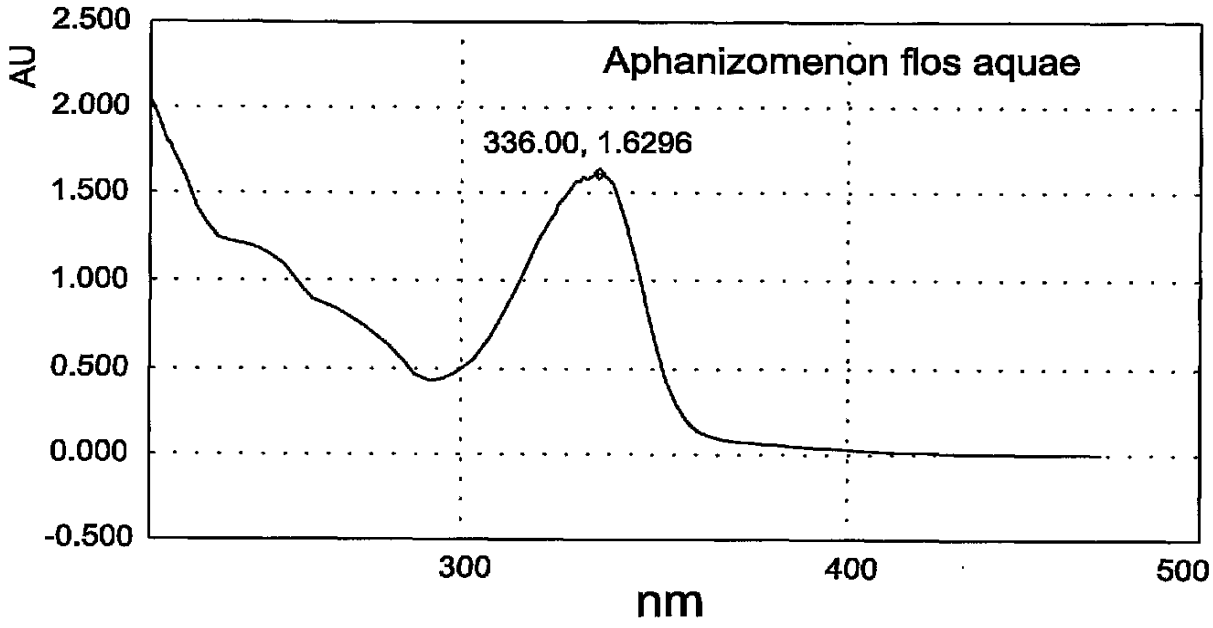

Aphanizomenon flos aquae
336.00, 1.6296
AU
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
300
400
500
nm

FIG. 1A

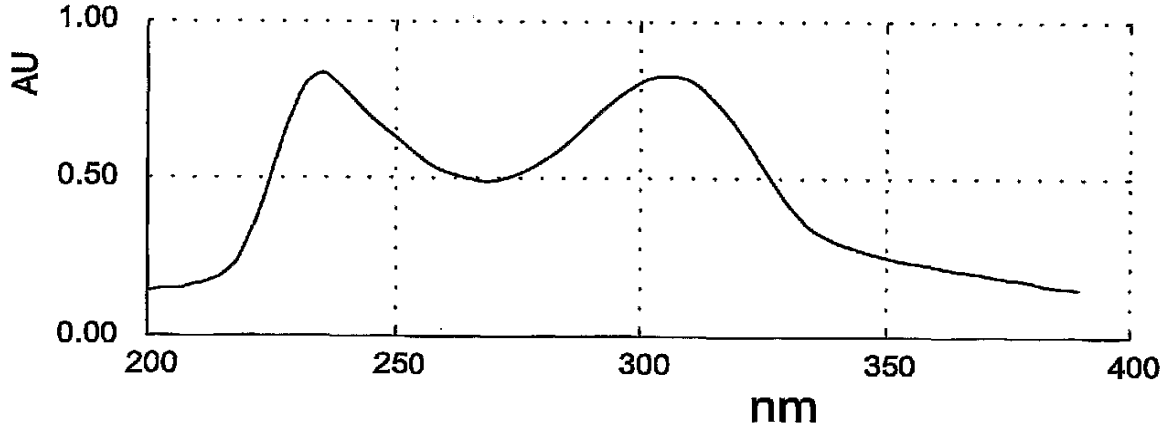

AU
1.00
0.50
0.00
200
250
300
350
400
nm

FIG. 1B

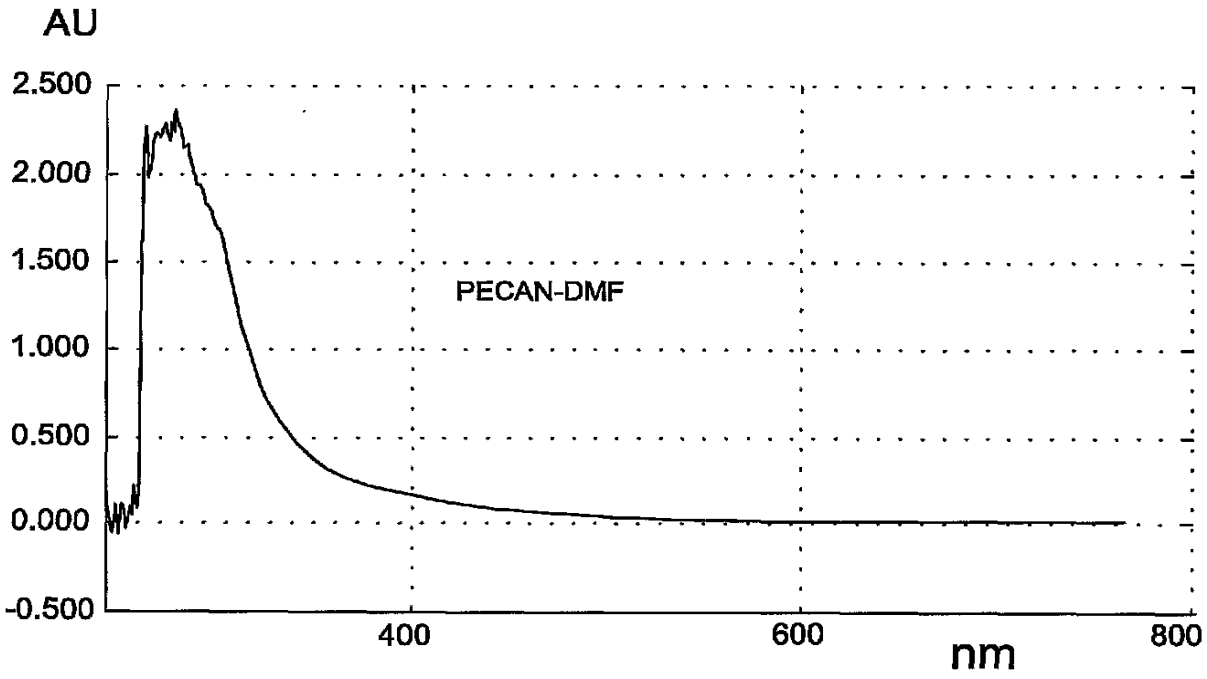

AU
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
PECAN-DMF
400
600
800
nm

FIG. 2A

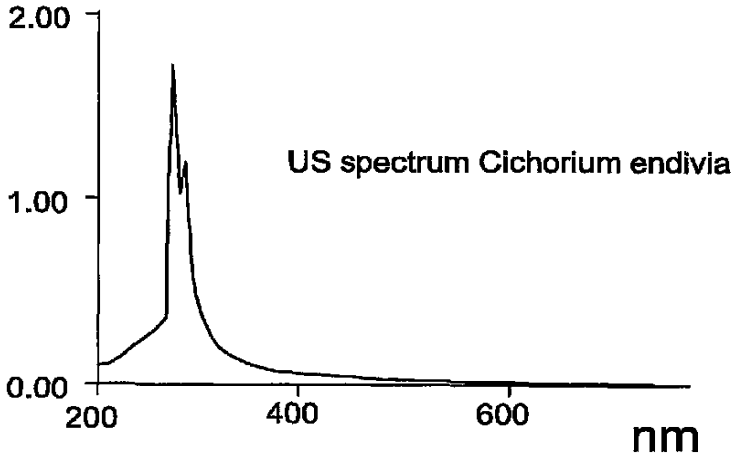

2.00
1.00
0.00
US spectrum Cichorium endivia
200
400
600
nm

FIG. 2B

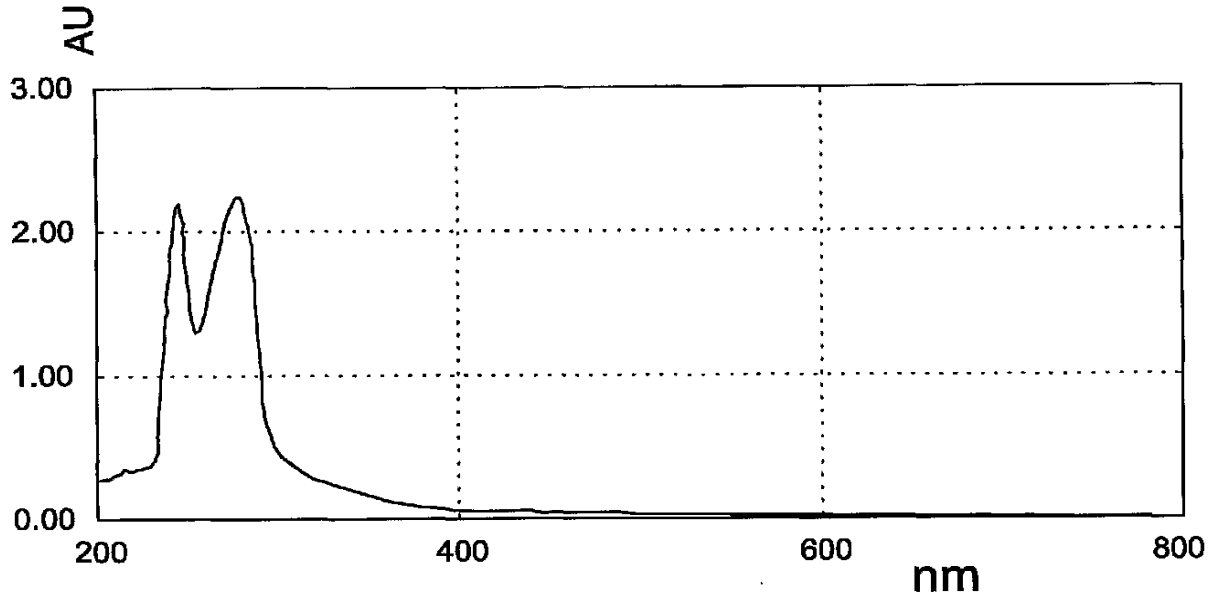

AU
3.00
2.00
1.00
0.00
200
400
600
800
nm

FIG. 3A

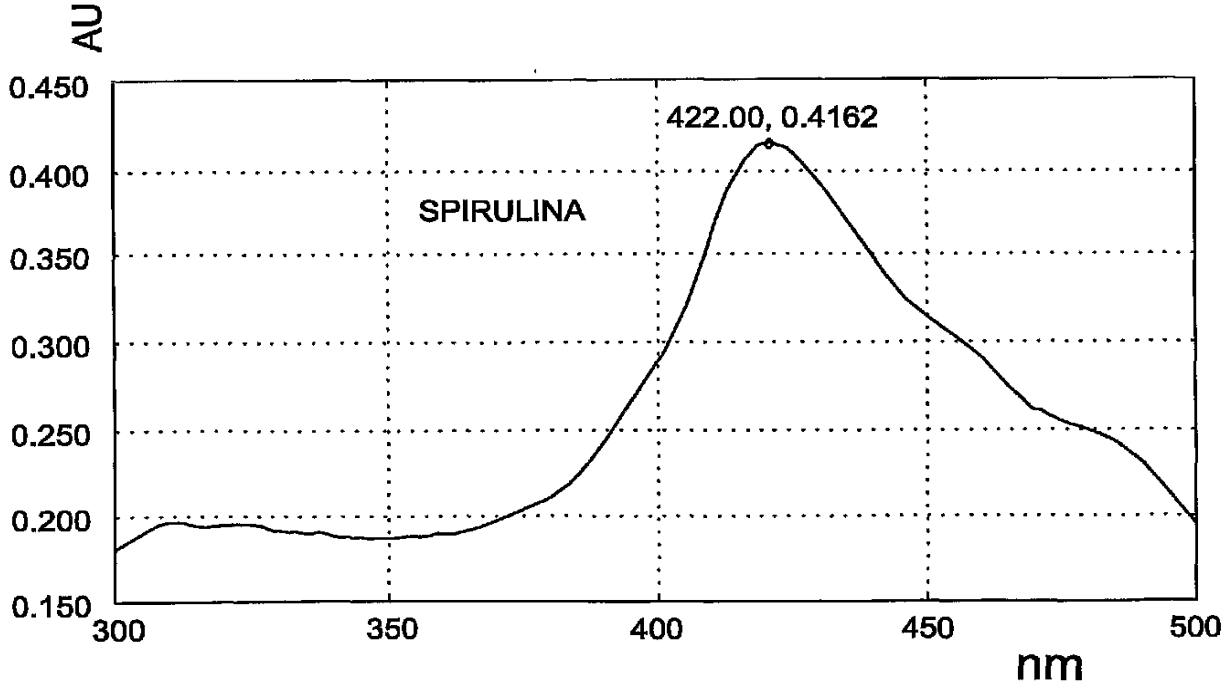

AU
0.450
0.400
0.350
0.300
0.250
0.200
0.150
422.00, 0.4162
SPIRULINA
300
350
400
450
500
nm

FIG. 3B

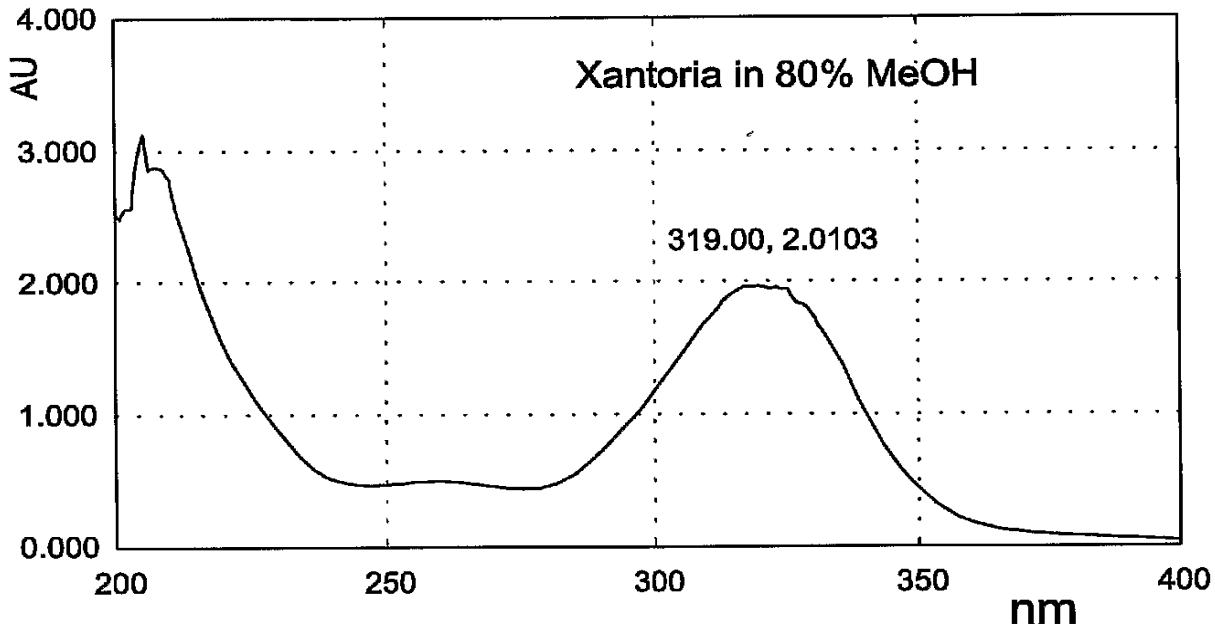

Xantoria in 80% MeOH
319.00, 2.0103
AU
4.000
3.000
2.000
1.000
0.000
200
250
300
350
400
nm

FIG. 3C

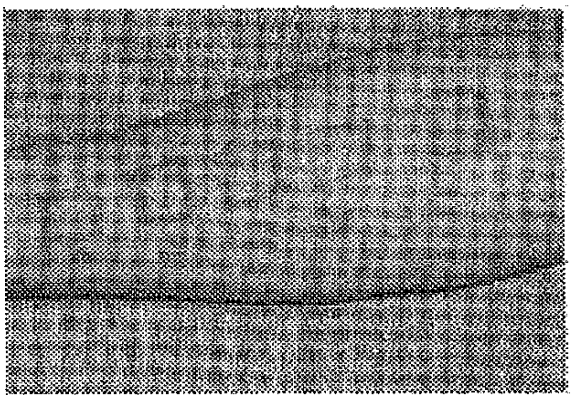


FIG. 4

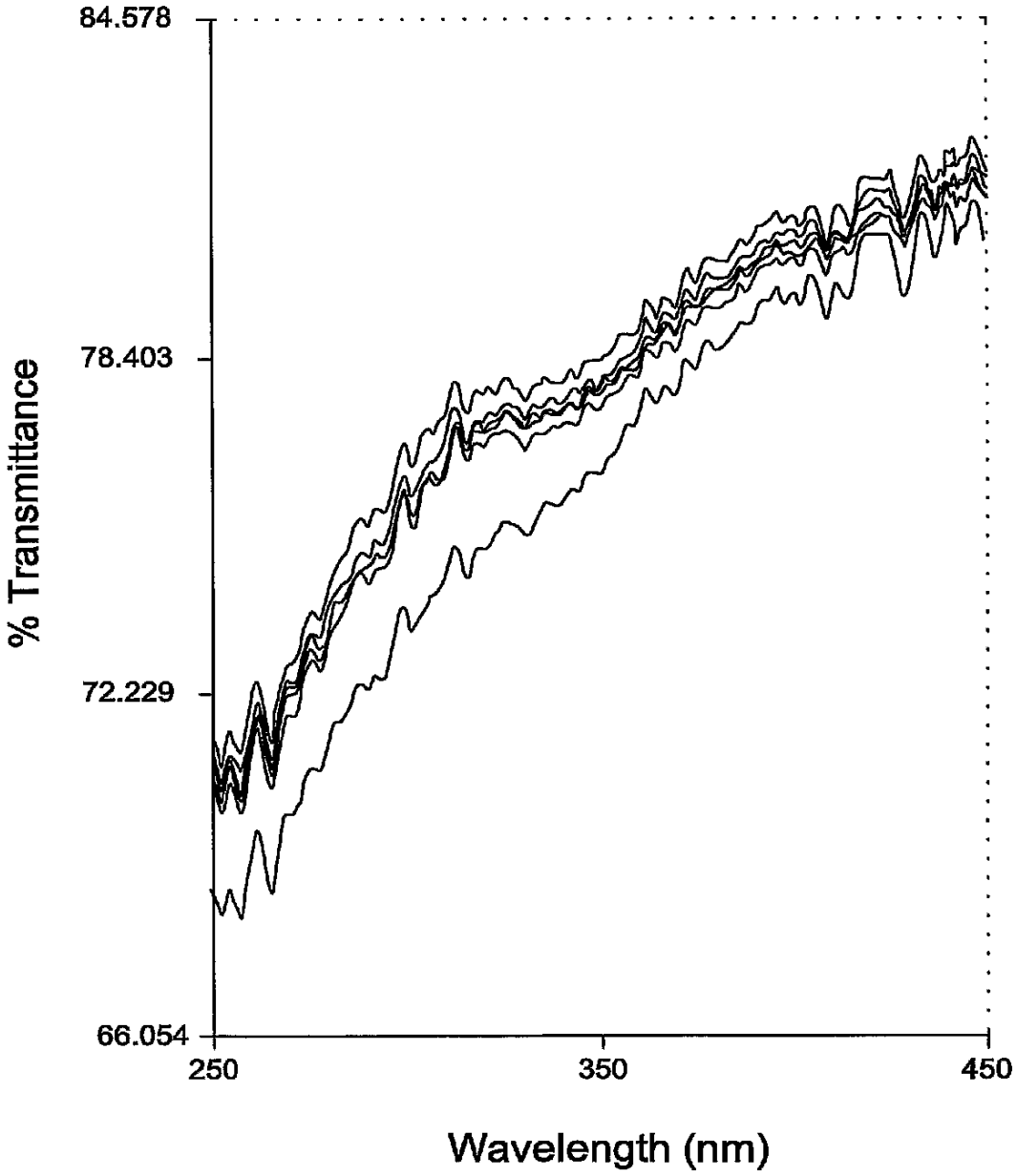

84.578
78.403
72.229
66.054
% Transmittance
250
350
450
Wavelength (nm)

FIG. 5A

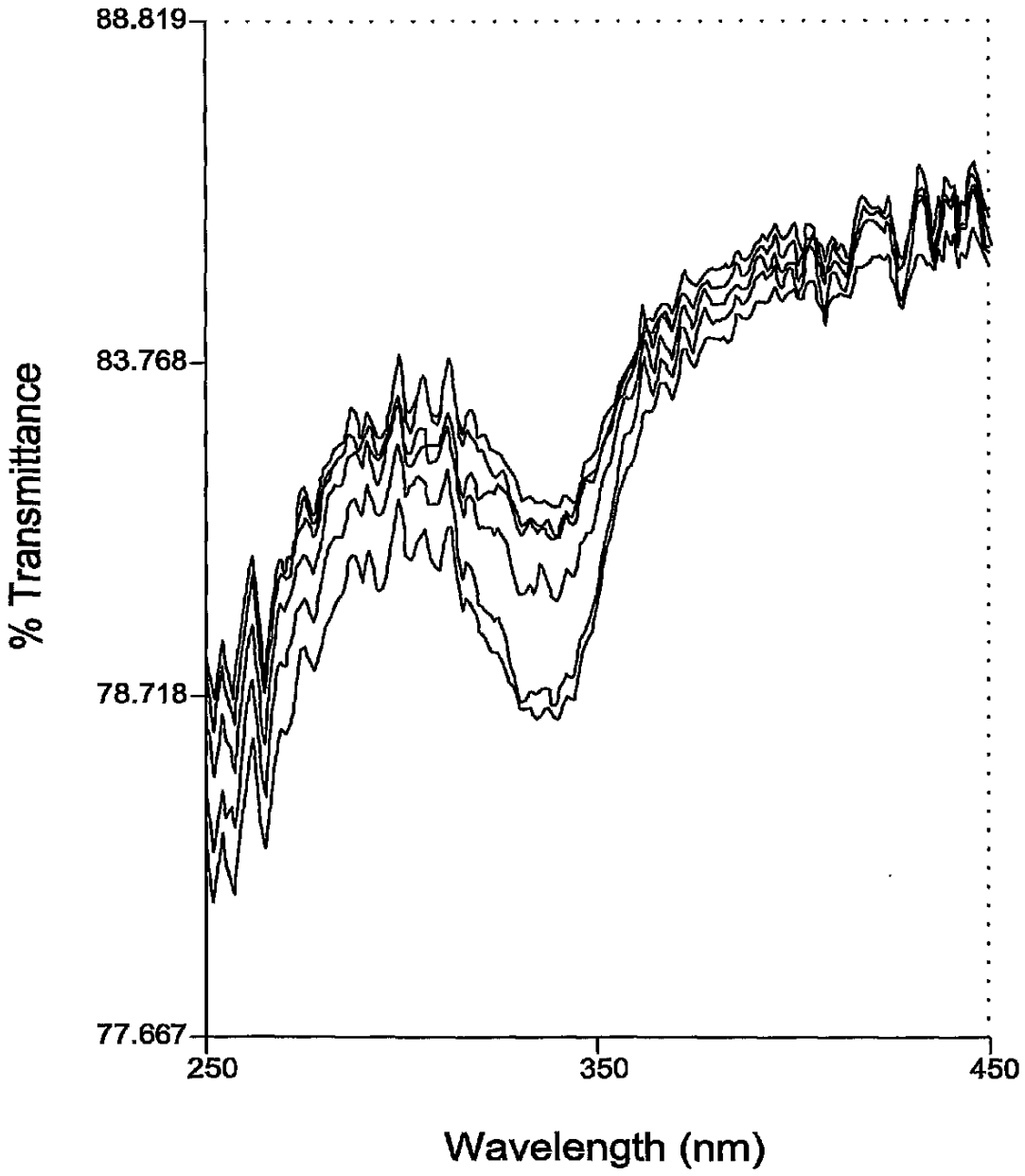

88.819
83.768
78.718
77.667
% Transmittance
250
350
450
Wavelength (nm)

FIG. 5B

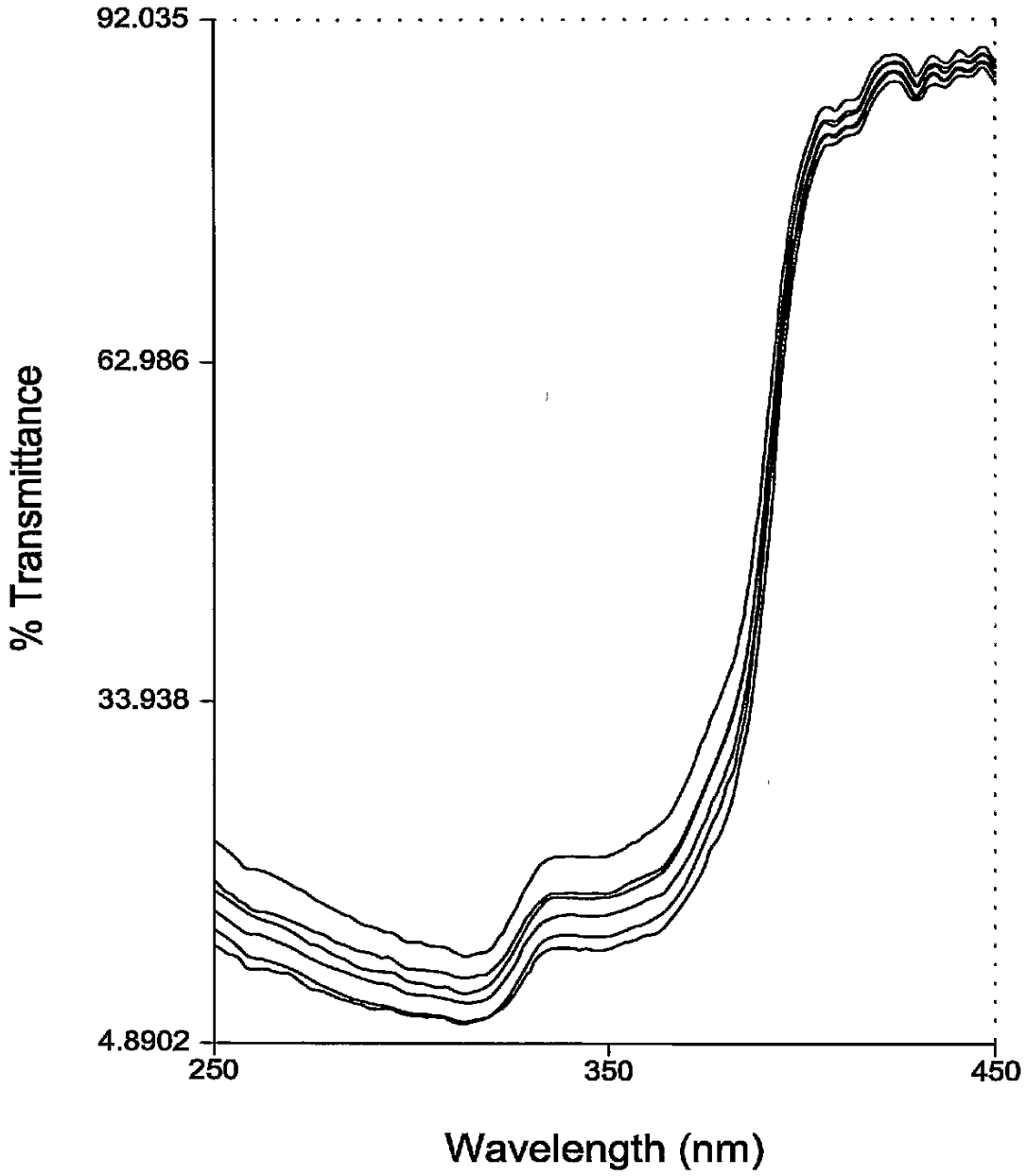

92.035
62.986
33.938
4.8902
% Transmittance
250
350
450
Wavelength (nm)

FIG. 6A

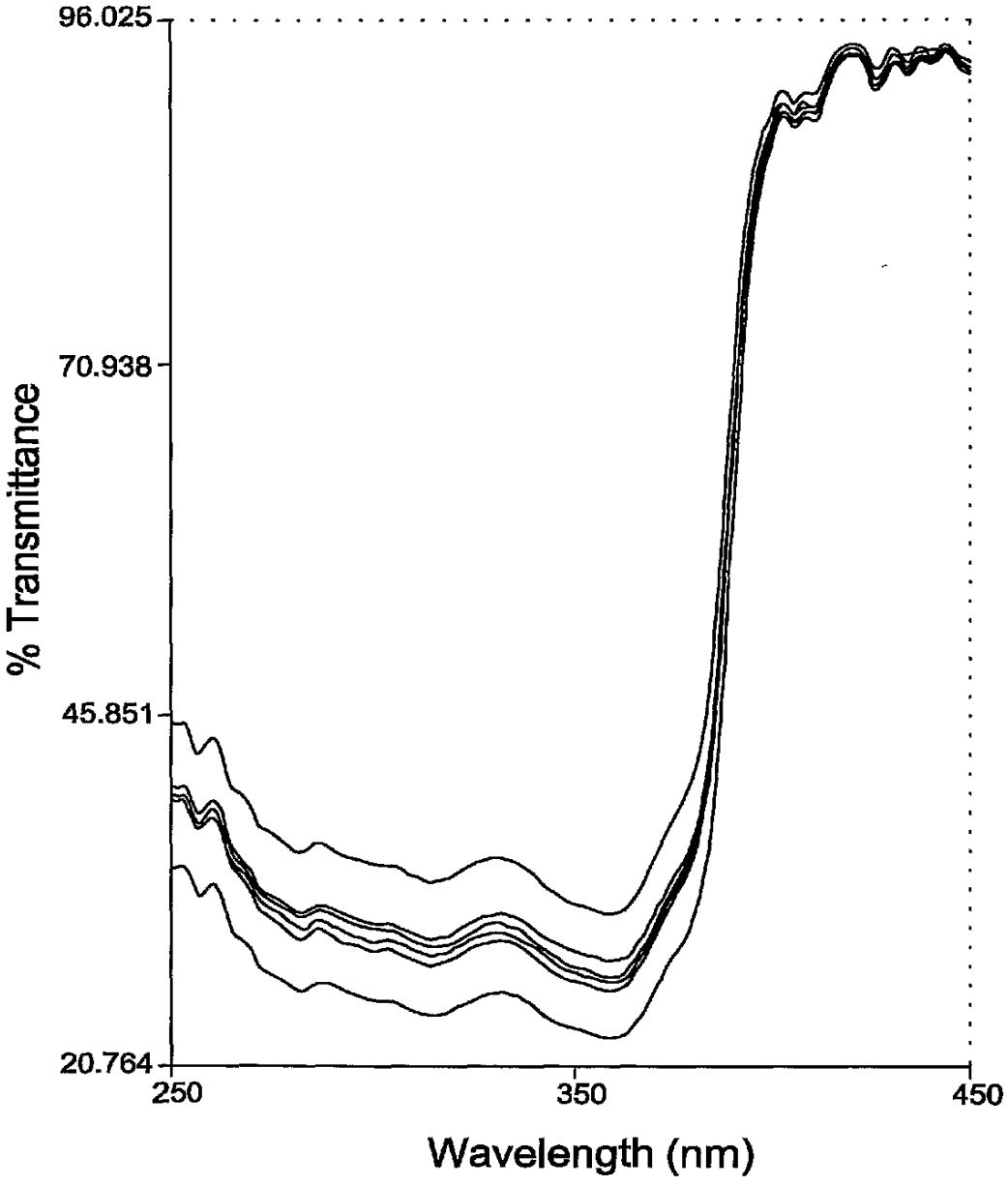

96.025
70.938
45.851
20.764
% Transmittance
250
350
450
Wavelength (nm)

FIG. 6B

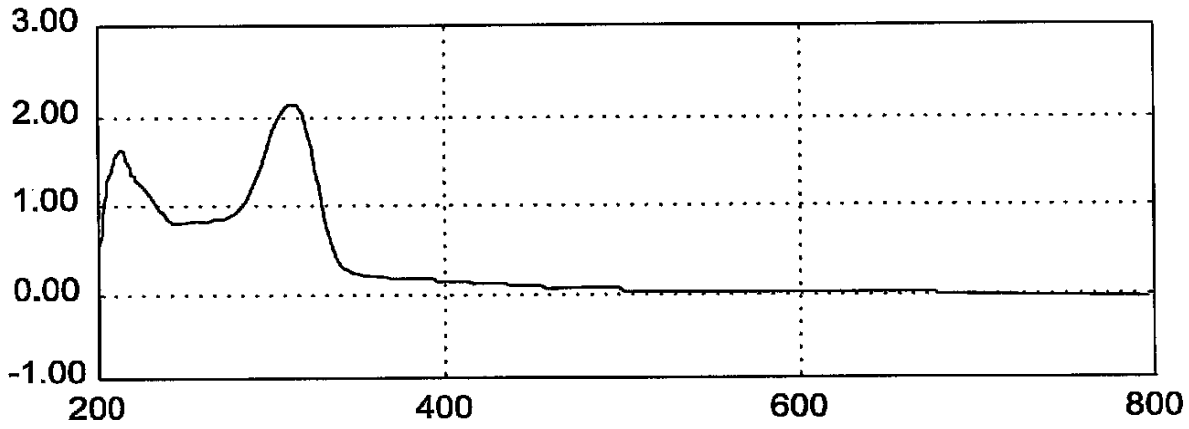


FIG. 7

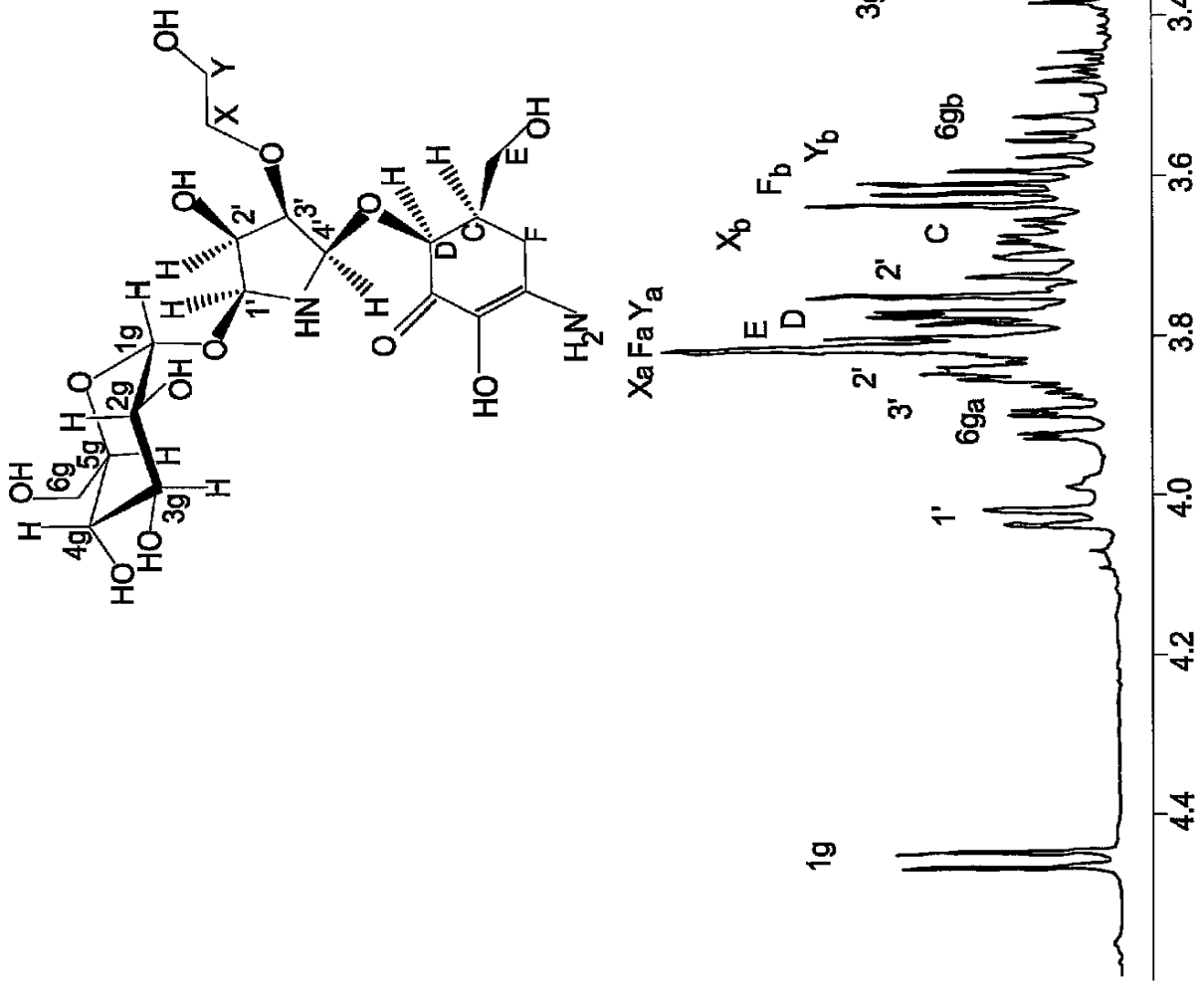


FIG. 8

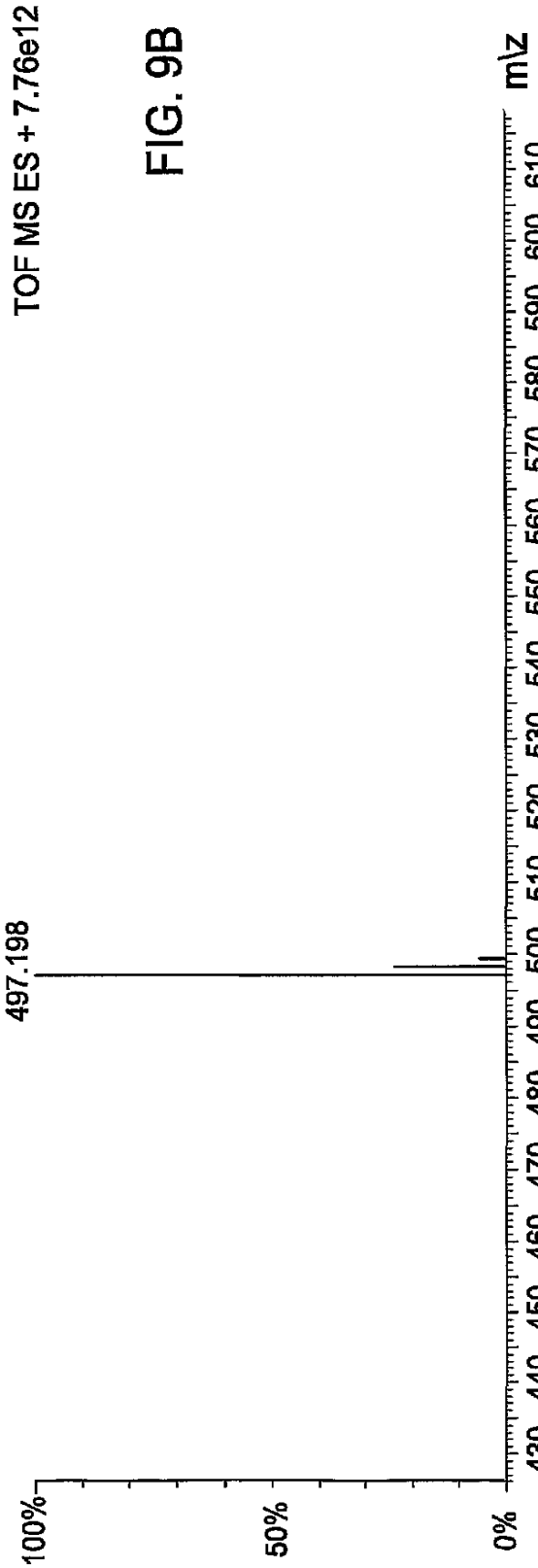

TOF MS ES + 7.76e12
497.198
100%
50%
0%
430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610
m\z

FIG. 9B

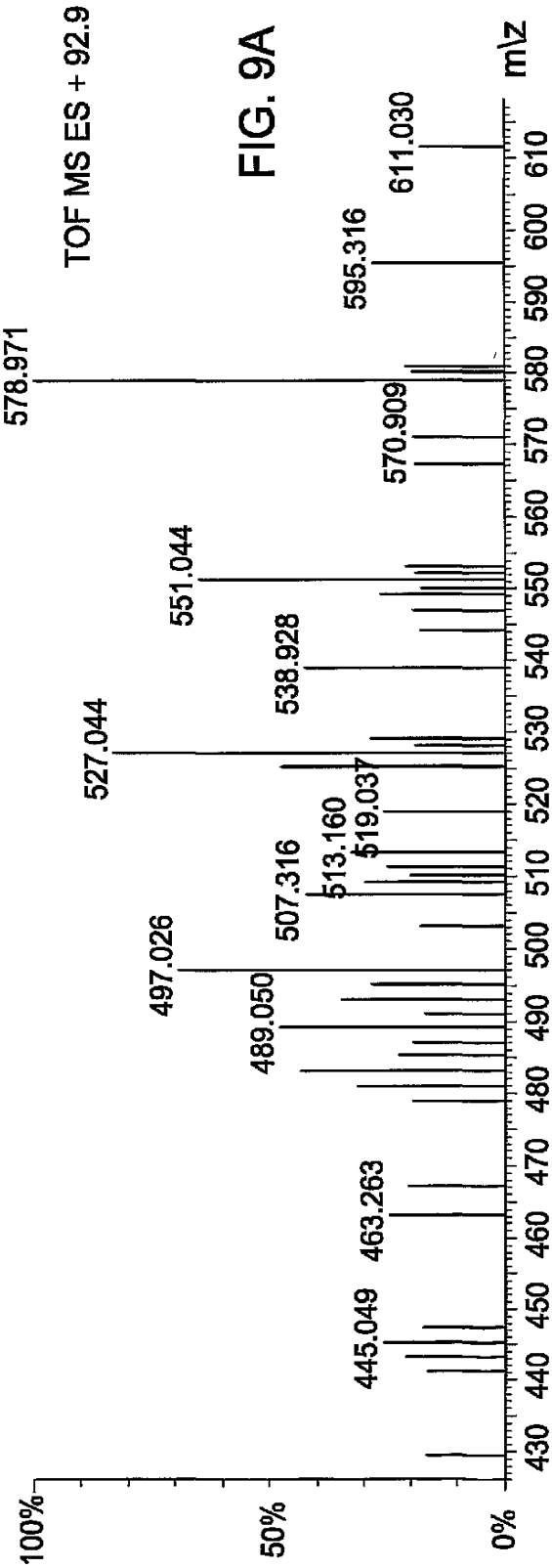

TOF MS ES + 92.9
578.971
595.316
611.030
570.909
551.044
538.928
527.044
519.037
513.160
507.316
497.026
489.050
463.263
445.049
100%
50%
0%
430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610
m\z

FIG. 9A

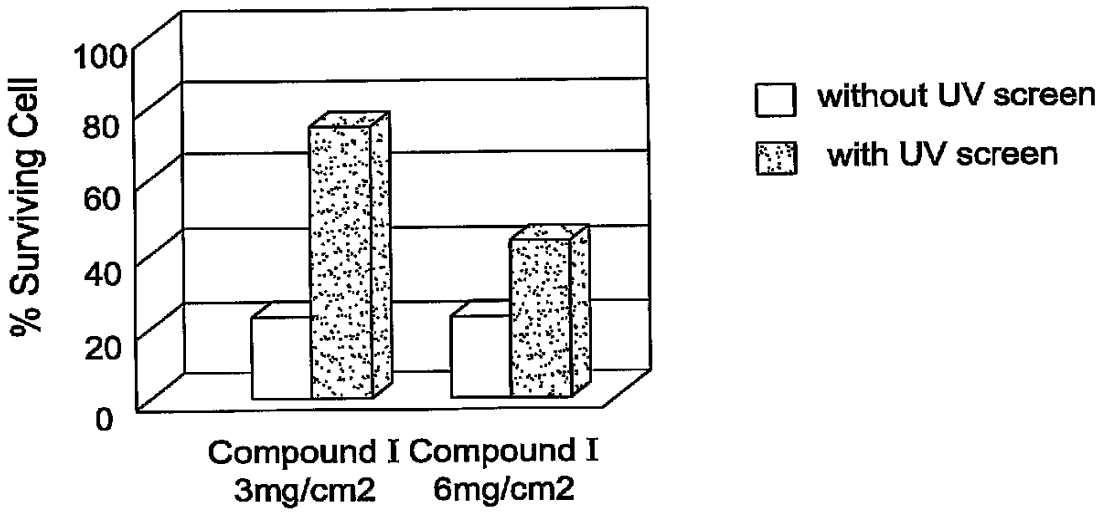


FIG. 10A

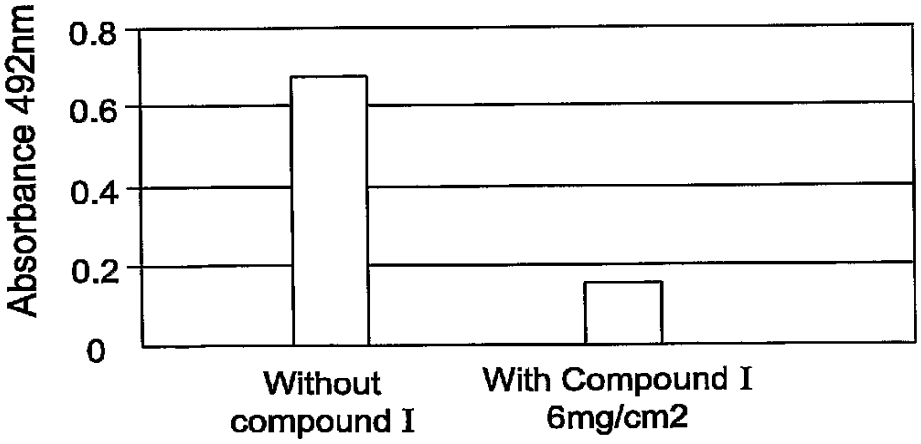


FIG. 10B

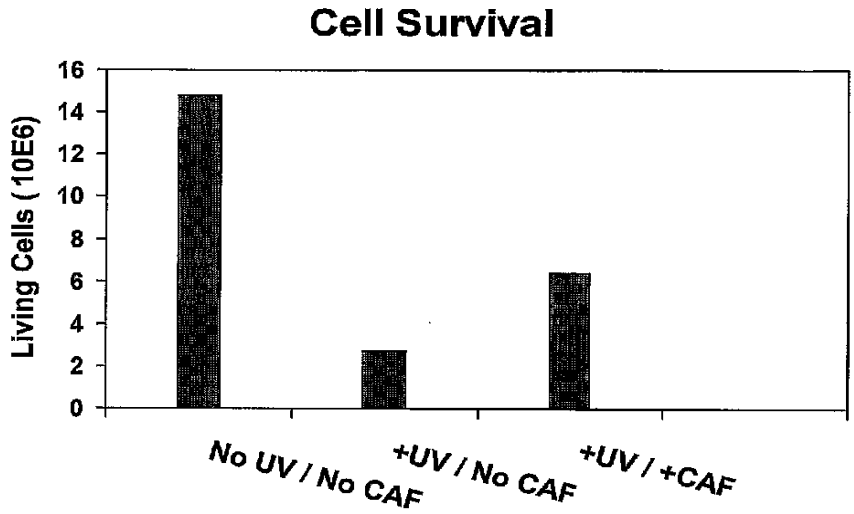


FIG. 11A$_{I}$

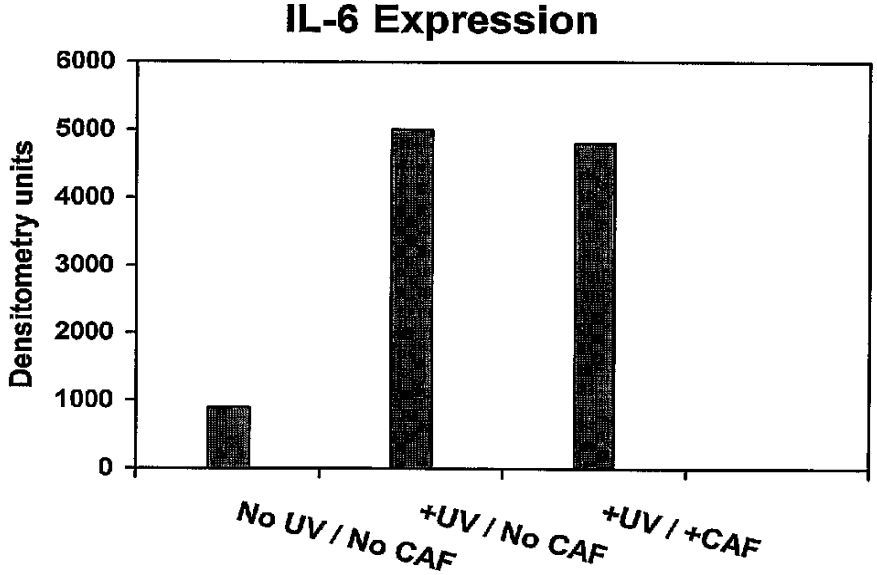


FIG. 11A$_{II}$

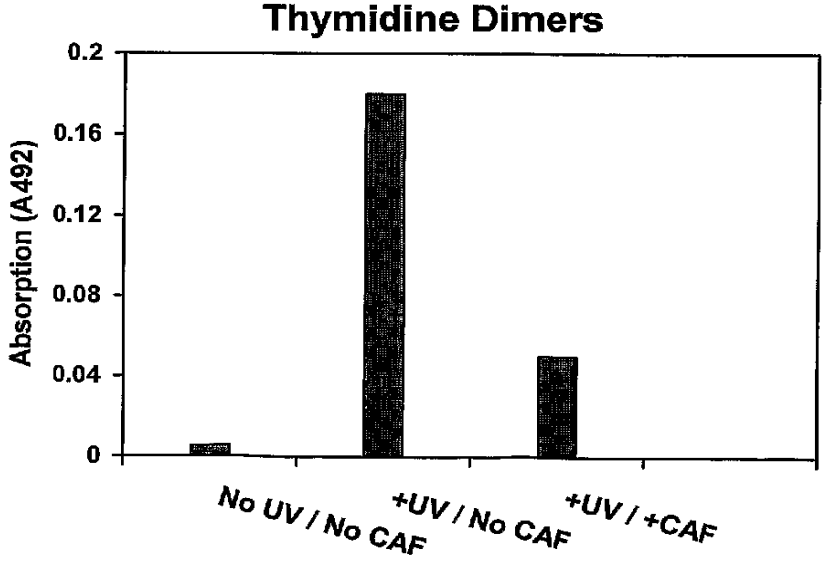


FIG. 11A$_{III}$

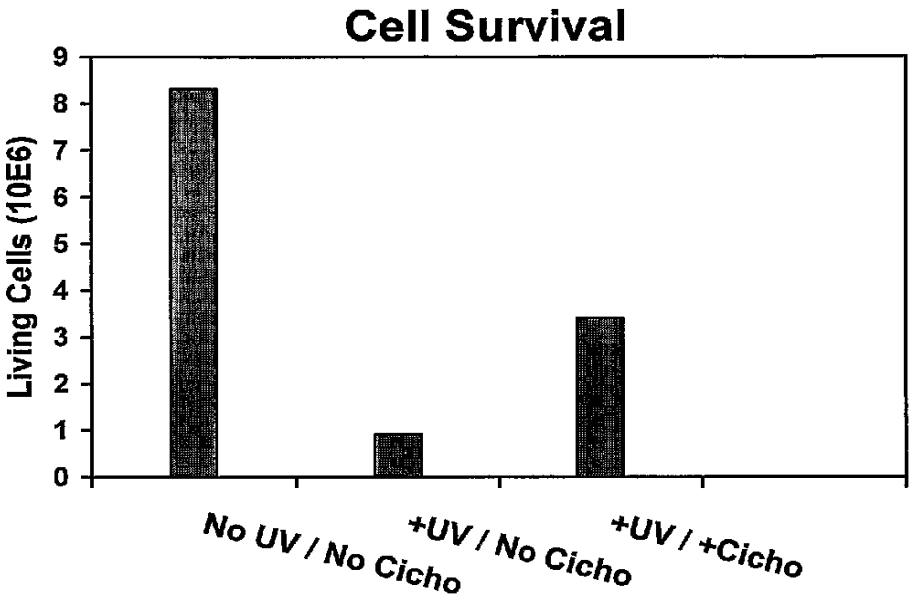


FIG. 11B$_{I}$

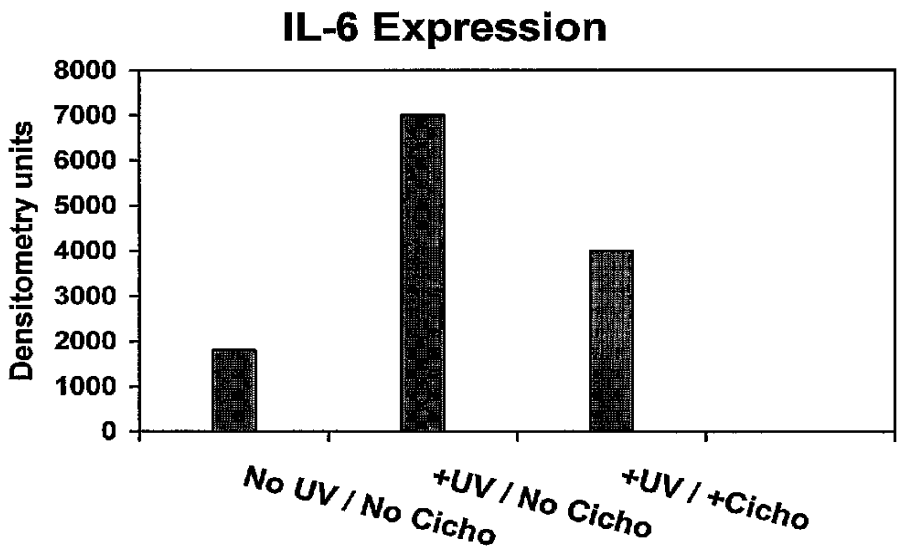


FIG. 11B $_{II}$

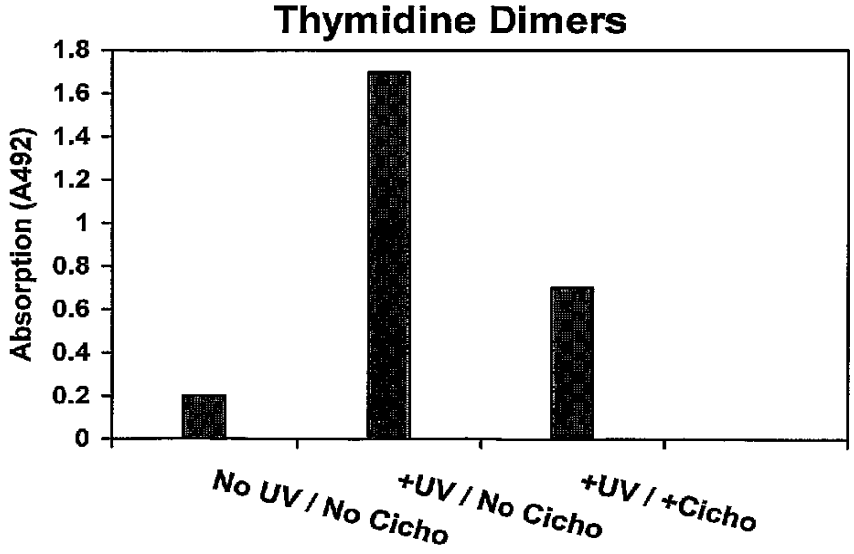


FIG. 11B $_{III}$

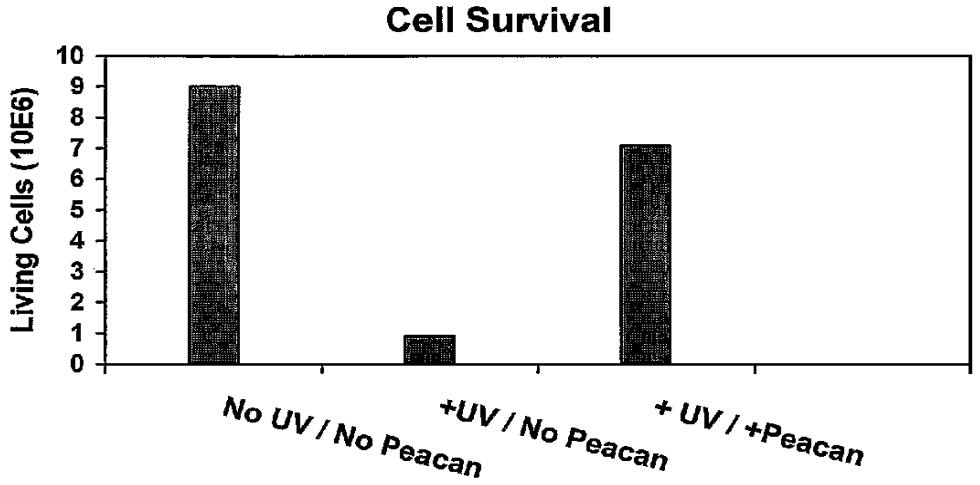


FIG. 11C I

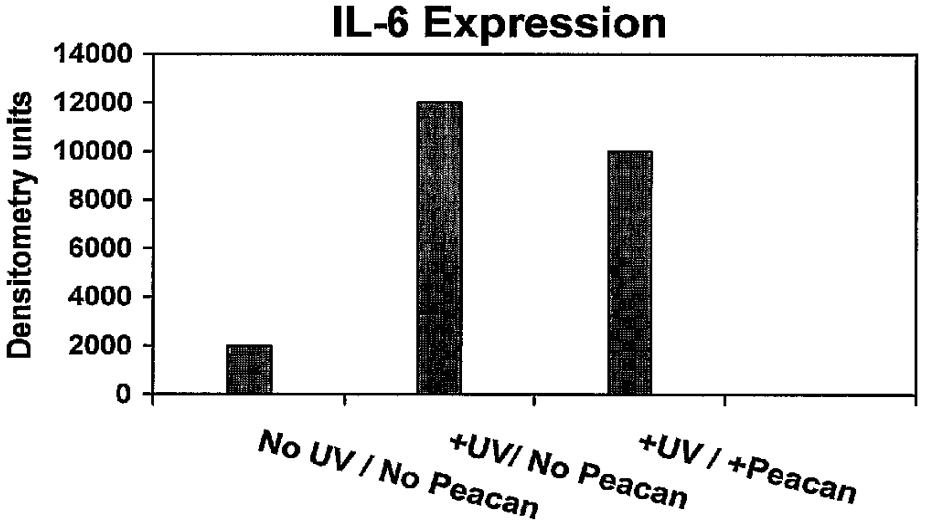


FIG. 11C II

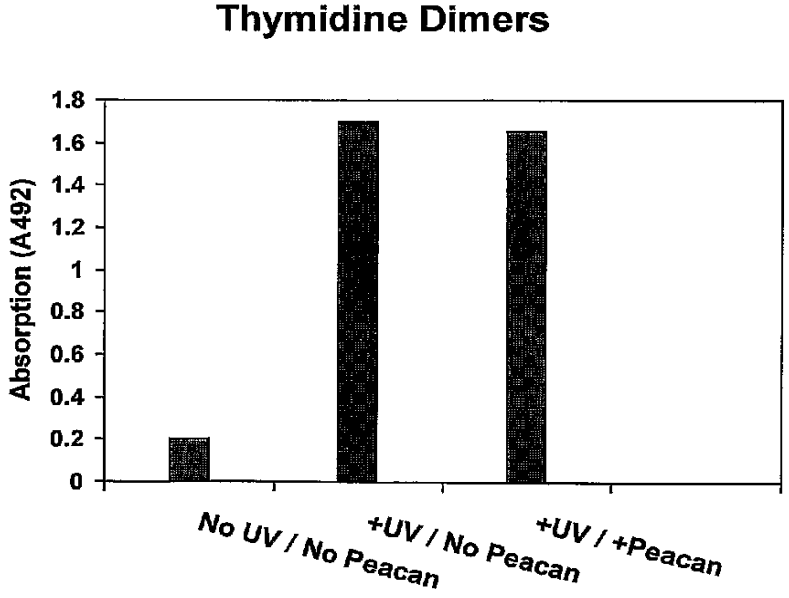


FIG. 11C III

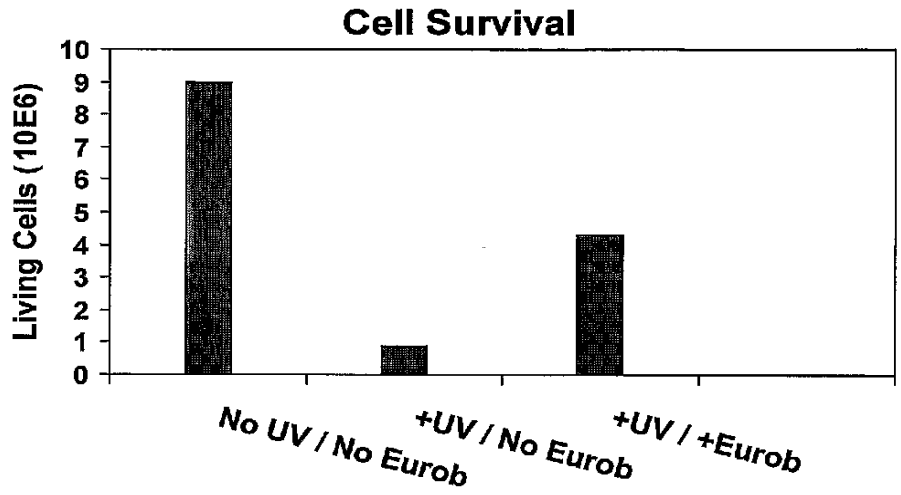


FIG. 11D I

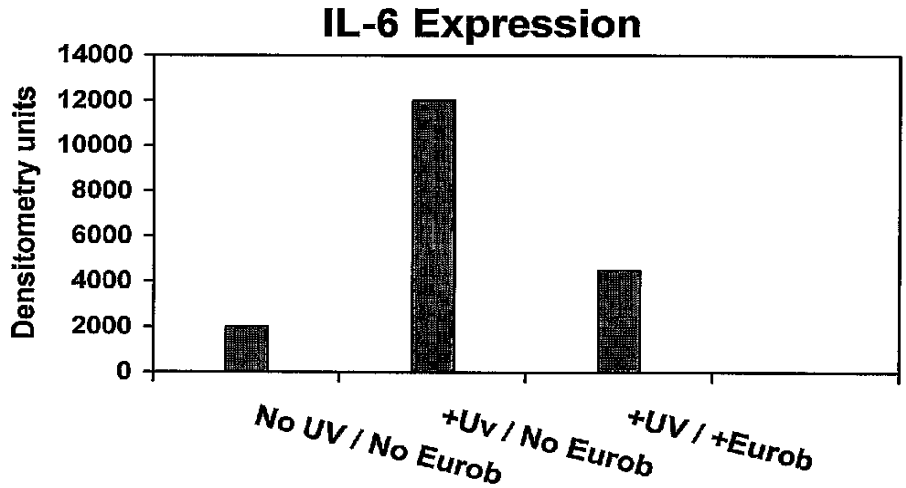


FIG. 11D II

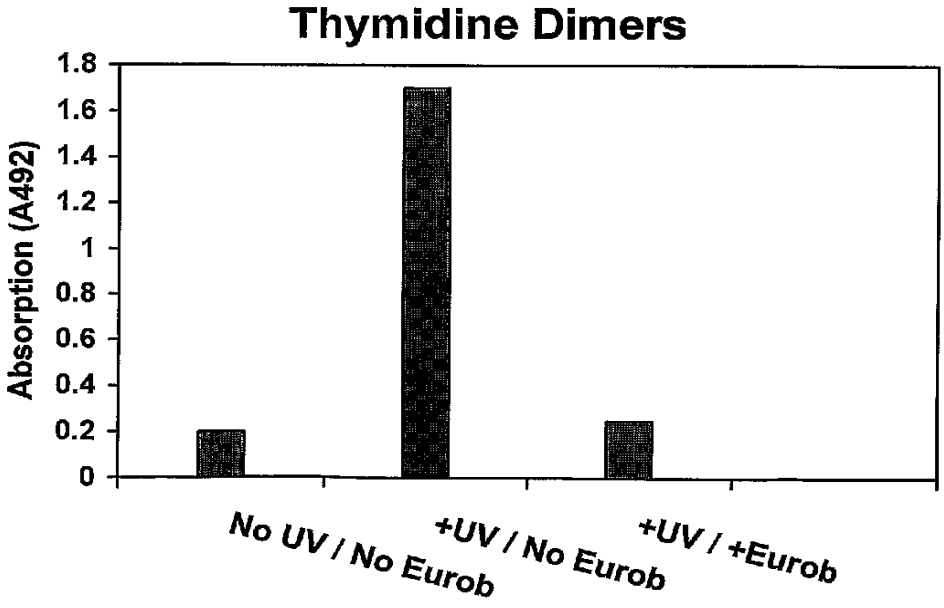


FIG. 11D III